Analysis of epigenetic regulation in prostate cancer: prognostic and therapeutic importance

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Synopsis

Prostate cancer (PCa) is a male specific malignancy and globally, it is ranked as the 2nd most common cancer among men.[1] PCa risk factors include advancing age, family history of cancer, body weight and obesity, smoking and alcohol habit, BMI, etc.[1-3] Some authors reported oncogenic HPV infection as a risk factor of PCa but others did not find any association of the virus with the disease that seems to make this issue a scientific debate.[4] The incidence of PCa is increasing gradually in developing countries like India. According to a Population Based Cancer Registries (PBCRs), in India from 2012–2016, prostate cancer became one of the most leading cancer type in major metropolitan cities like Delhi, Mumbai, and Bangalore, Kolkata and so on.[5] Thus, on the background of increased trend on prostate cancer incidence in our country, potent healthcare strategies need to be developed for effective disease management.

In PCa, digital rectal examination (DRE) and serum Prostate Specific Antigen (PSA) testing are recognized as keystone diagnostic tools; but, both methods have limitations, as having low sensitivity and specificity.[6-7] Transrectal Ultrasound Scan (TRUS)-guided biopsy is considered as a gold-standard protocol for prostate cancer diagnosis and the prognosis is predicted by Gleason scoring, which is a microscopic evaluation method, and therefore often subjected to inter-observer variability.[8-9] Therefore, situation warrants identification of molecular markers that can be implicated to improve the existing diagnostic and prognostic measures substantially and for this, a detailed study on the disease pathogenesis at molecular level is needed.

Although, alterations like 8p deletion, TMPRSS2-ERG fusion were reported frequently during prostate tumorigenesis, but presence of huge intra-tumoral genetic variation (variation between different foci of same tumor) did not seem to suggest the genetic origin of prostate cancer.[10] Epigenetic alterations including DNA methylation and histone modification were reported as early events in prostate carcinogenesis.[10] Among histone modifications, aberrant changes in histone methylation and dysregulation of associated modulators were studied frequently in PCa.[11-14] Transcriptional modulation of genes by promoter methylation is usually regulated by coordinated and opposing action of DNA methyl transferase (DNMT3A &3B, de-novo methylase; DNMT1, maintenance methylase) and DNA demethylase

(Ten eleven translocase protein, TET1, TET2 & TET3) enzymes under normal condition.[15] In prostate cancer, aberrant high expression of DNMT1 and reduced/loss of expression of TET1 and TET2 were reported separately in different investigations indicating the incidence of DNA epigenetic dysregulation and its importance in disease development.[16-19] But, the underlying molecular mechanisms for aberrant expression of DNMT1, TET1, and TET2 was not studied in details, and in TET3, no study has been conducted in relation to PCa development to date. Apart from DNA methylation, the role of histone modification, specifically lysine methylation maintained by histone lysine methyl transferase (KMTs) and histone lysine demethylase (KDMs) was extensively studied in cancer,[20] and it seems to have complex impact on gene expression i.e. methylation at certain lysine residues (H3K4, H3K48 and H3K79) was associated with gene activation while that at others (H3K9, H3K27) with gene silencing.[21] The dysregulation of KMTs and KDMs leading to aberrant change in transcriptional network was reported in cancer and, the candidature of these enzymes to be targeted by potential inhibitors for therapeutic purpose is tested in ongoing research.[22-23] Among KMTs, euchromatin histone lysine methylase 2 (EHMT2/ G9a) involves in methylation of a repressive histone mark H3K9, and is reported to be over-expressed in solid tumors including PCa;[24-28] but, its genetic/epigenetic alterations remain relatively less explored in cancer.

Like EHMT2, DOT1L is also overexpressed and plays an oncogenic role in solid tumors,[29-31] but studied rarely in prostate cancer.[32] Among KDMs, PHD finger protein 2 (PHF2) and lysine specific demethylase 2B (KDM2B) demethylates repressive (methylated H3K9) and active histone marks (H3K4, H3K36 & H3K79) histone marks respectively leading to transcriptional activation and silencing of target genes respectively. [33-34] Tumor suppressor function of PHF2 was reported in malignancies of other organs,[33,35-36] but no study yet conducted in prostate cancer. The role of KDM2B in tumorigenesis remains controversial as it plays both oncogenic and tumor suppressive roles in different malignancies.[37]

Thus, in PCa, the dysregulation of epigenetic modulators seems to be a frequent event; this obviously results into global epigenetic changes leading to aberrant activation of oncogenes and inactivation of tumor suppressors that ultimately results into dysregulation of vital cellular pathways like cell cycle checkpoint, DNA damage response etc. contributing significantly to the disease pathogenesis. Thus, to

understand the molecular pathogenesis of PCa in details, present study aimed to investigate the association of oncogenic HPV as an epidemiological factor at first, then analyse the alterations of potential epigenetic modulators as well as explore the impact on global epigenome, and lastly, assess the impact of aberrant epigenetic changes in the dysregulation of some vital cellular pathways (cell cycle checkpoint/DNA damage response) relevant to the disease development/progression/prognosis, in a sample set of normal prostate (expressed prostatic fluid/EPS), benign lesions (BPH) and malignant tumors (PCa); no such comprehensive study has yet been done to our best. Such integrated, in-depth analysis would be helpful to identify disease associated molecular markers of clinical importance.

In our studied samples (control, BPH and PCa), oncogenic HPV infection showed a significantly increasing trend from control to prostatic lesion; however, among the lesion samples, the frequency remained comparable between BPH and PCa. Therefore, consistent to the finding of many other investigators,[4] we too failed to establish the association of oncogenic HPV infection with PCa development. However, our data suggested a potential role of the viral infection with development of benign prostatic lesions that needs to be validated in independent studies.

Next, we studied the alterations (expression and associated alterations) of the DNA and histone epigenetic modulators, and its impact on the global epigenome. To select DNA and histone epigenetic modulators for expression analysis, epigenetic alterations of potential candidates was analysed first. Among DNA epigenetic modulators, DNMT1 showed a significantly decreasing trend, while TET1 and TET2 showed a significantly increasing trend of promoter methylation from normal prostate to BPH to PCa, suggesting their epigenetic dysregulation during prostate tumorigenesis. But, in TET3, decrease of methylation frequency from BPH to PCA after its steady increase from normal prostate to BPH was seen; thus, alteration of TET3 seems to be associated with the development of benign lesions. Consistent to epigenetic alterations, DNMT1 showed over-expression and TET1 and TET2 expression level was reduced at RNA and protein levels in PCa compared to BPH and/or normal prostate. Similar to our findings, DNMT1 over-expression and reduced TET1 and TET2 expression was seen in PCa.[16-19] However, significant association of promoter hypermethylation of TET1 and TET2 with their reduced

expression as evident in our study, indicated epigenetic mode of inactivation of these candidates. Also in our study, a significant association of DNMT1 protein with promoter methylation of TET1 and TET2 indicated the role of the former in epigenetic inactivation of later. To assess the dysregulation of DNA epigenetic modulators, the content of epigenetically modified base (5hmC) was analysed by immunohistochemistry. In our sample set, 5hmC content was found to be reduced in PCa samples compared to BPH, and additionally, showed a positive correlation with the TET1 and TET2 proteins and negative correlation with DNMT1. Similar to us, the functional impact of dysregulation of TET1 and TET2 on 5hmC content of genome was reported in independent studies;[17,19] but in our study, the impact of DNA methylase and de-methylase on 5hmC content was assessed on same sample set, which has not previously been done to our best. In our studied patient population, bone metastasis (BM) was found as the most frequent adverse disease outcome. Interestingly, methylation status of TET1 and TET2 combinedly found as an independent and significant predictor of hazardous patient outcome with respect to BM.

Among histone epigenetic modulators, our study focused on selected histone lysine methyl transferase (KMTs; EHMT2, DOT1l) and histone lysine demethylase (KDMs; PHF2, and KDM2B) based on their association with different malignancies, but unavailability/ insufficient information on their role in PCa pathogenesis.[24-37] However, among the potential histone epigenetic modulators, only EHMT2, as showing a significant decreasing trend of promoter methylation from normal prostate to BPH to PCa, was selected for the expression analysis. EHMT2 showed a significant mRNA level over-expression in BPH vs. normal prostate, and importantly, in PCa vs. BPH. High level of EHMT2 protein was also found in PCa compared to BPH. Consistent to our finding, EHMT2 over-expression was reported in PCa in one study.[28] But the associated molecular mechanism remained unexplored. In hepatocellular carcinoma, copy number gain at EHMT2 locus was found and suggested to contribute to the gene over-expression.[38] But apart from genetic alterations like copy number variation and point mutation, epigenetic alterations like promoter hypomethylation may also contribute to the activation of oncogenes.[39-40] In our study, a decreasing trend of EHMT2 promoter methylation from normal prostate to BPH to PCa, and, a significant inverse association of its mRNA and protein level

expression with promoter methylation suggested the contribution of epigenetic mechanism in up-regulation of the gene during prostate carcinogenesis. A significant correlation of EHMT2 protein in PCa with H3K9Me2 content of the genome was also found, indicating the functional impact of the gene over-expression on global epigenome. Thus, EHMT2 together with H3K9me2, which is a repressive histone mark, might lead to transcriptional silencing of TSGs during prostate carcinogenesis. Interestingly, both EHMT2 protein level and H3K9me2 content showed a significant associated with the advanced disease clinicopathological parameters. Additionally, it was observed that patients having high expression level of EHMT2 showed poor bone metastasis-free survival. These suggested the prognostic importance of EHMT2 in PCa patients.

In our study, dysregulation of DNMT1, TET1, TET2, and EHMT2 were found, the functional impact of their dysregulation on global epigenome was evident. According to our hypothesis, the dysregulation of epigenetic modulators during prostate carcinogenesis might cause aberrant promoter hypermethylation and transcriptional silencing of important tumor suppressors leading to dysregulation of vital cellular pathways, of which, cell cycle checkpoint and DNA damage response (DDR) are considered in the present study. We did a comparative analysis of whole genome methylome data between normal prostate vs. BPH vs. PCA of our sample set to identify differentially hypermethylated genes in prostatic lesions compared to control. Using GDC-TCGA PRAD dataset (publicly available data set on PCa), the downregulated genes in prostatic lesions were screened, and, overlapped with hypermethylated genes obtained in our dataset to identify hypermethylated as well as downregulated gene set. Next, hypermethylated and down regulated genes were subjected to pathway analysis, and 45 differentially methylated genes (DMGs) associated with 9 DNA damage response (DDR) pathways were identified. 34 genes among 45 candidates as showing significantly increasing trend of promoter methylation from control to BPH to PCA, were considered as important contributor to the development of malignant prostate. Among 34 DDR pathway genes 6 candidates showed a significant promoter hypermethylation in PCa compared to BPH within the lesions, hence seemed play role in prostate carcinogenesis. Among 6 genes, epigenetic alterations of SPATA-18 (spermatogenesis associated 18) or MIEAP (mitochondria-eating protein)[41-43] and IER3 (immediate early response 3)[44-

[46] are known to disrupt the mitochondrial homeostasis and cell cycle arrest, and NUAK1 (novel (nua) kinase family-1),[47-49] APC,[50-51] REC8[52-54] plays a role in DNA damage repair, cell cycle arrest, and/or apoptosis, disruption of which might lead to error-prone DNA repair and tumor development. Although the functional role of these genes, except APC, is characterised in malignancies other than prostate, the epigenetic alteration of these DDR genes associated with the progression of prostate cancer is first ever studied to the best of our knowledge.

Concluding from this, in our study, overexpression of DNMT1 & EHMT2, and transcriptional silencing of TET1 & TET2 provided evidence for incidence of epigenetic dysregulation in prostate tumorigenesis. The knowledge incurred seemed to provide important information for understanding disease pathogenesis. The significant association of TET1, TET2, and EHMT2 with bone metastasis indicated their prognostic importance. Therefore, molecular alterations (promoter methylation/ expression) of TET1, TET2, and EHMT2 in tissue biopsy may be used for predicting patient outcome with an aim to improve existing prognostic measures. Also, in our methylation array data, potential association of 6 DNA damage response (DDR) genes with PCA pathogenesis was found out of which no study has been conducted in PCa with 5 candidates to date. Therefore, considering the importance of DDR pathway in tumorigenesis and in modulation of tumor response to radiotherapy, further research is warranted on these candidate genes to validate their role in PCa development and explore their clinical relevance.

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